K. A. Mereish

INTERACTION OF CYCLIC PEPTIDES

AND DEPSIPEPTIDES WITH CALMODULIN

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A variety of small peptides including hormones, neurotransmitters, and insect venom components such as ACTH, β-endorphin, and melittin, respectively, bind calmodulin (CaM) and inhibit CaM-dependent enzyme activity (1). The binding has been shown to be of high affinity; saturable; calcium dependent; and, upon the removal of calcium, reversible. Peptides bind CaM in either a 1:1 or 1:2 molar ratio and can be competitively displaced from CaM by other non-peptide CaM antagonists such as phenothiazines (2). Structure-activity studies have demonstrated that most CaM-binding peptides possess either basic amino acids, hydrophobic amino acids, or alpha helical secondary structure (3,4). Alpha helical secondary structure was thought to be a necessary requirement in the recognition and binding processes, since the peptides that bind the strongest to calmodulin - mastoparan and melittin - have marked propensities to form alpha helixes in solution (4). However, it has been shown that at least three peptides, gramicidin-S (GRS), ACTH, and dynorphin, which do not form alpha helixes, bind CaM with high affinity (1). These data collectively suggest that the CaM-binding peptides investigated so far possess at a minimum, hydrophobic residues, with some peptides having amphipathic or sided structures where hydrophobic and hydrophilic amino acid residues are located on opposite sides of the molecules (5).

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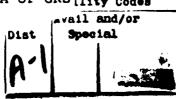
The cyclic peptides cyclosporin A (CSA) and GRS have been shown to bind CaM and inhibit bovine brain 3', 5'-cyclic nucleotide phosphodiesterase (PDE) in a calcium-dependent manner (6,7). We investigated the ability of other cyclic peptides: microcystin-LR (MLR) and depsipeptides, valinomycin (VLM) and enniatin-B (ENB), to bind dansylated CaM and inhibit PDE. Dansyl-CaM, which possesses the same physiochemical and functional properties as native CaM (8), has been used as a reliable indicator of calmodulin binding to calcium, protein, and peptides (9,10).

Upon the binding of calcium to CaM, the fluorescence of dansyl-CaM undergoes both an increase in intensity and a "blue shift" of the absorption maximum (λmax). Addition of peptides or proteins known to bind CaM produces a further blue-shift of λmax and/or an increase in fluorescence intensity (9,10). Changes observed in the fluorescence intensity and the blue shift of dansyl-CaM are believed to be due to an increase in the hydrophobicity of the environment in the vicinity of the fluorophore. The increase in hydrophobicity results in a higher quantum yield and higher energy λmax of the dansyl group (8). These fluorescence studies have been used to provide binding constants for several CaM-binding peptides and proteins (10). Therefore, we compared the fluorescence emission spectra of dansyl-CaM as a function of increasing peptide concentration in the prescence or absence of calcium (Fig. 1a-e).

In the presence of calcium, the fluorescence intensity of dansyl-CaM increased with increasing concentrations of CSA or GRS

(Fig. 1.a,b). The binding of CSA or GRS to dansyl-CaM also induced a blue shift of λmax. These spectral changes were not observed, however, if EGTA was present in the buffer (Fig.1), indicating that the binding of CSA or GRS to dansyl-CaM was calcium dependent and reversible (6,7). The increase in dansyl-CaM fluorescence intensity, as well as the blue shift of λmax induced by both CSA and GRS, is similar to the spectral changes induced by many of the CaM binding peptides and proteins; which suggests that they may bind to a common site. Binding constants (Kd) of 4.8 μM and 2.85 μM were estimated for CSA and GRS, respectively, by using the method of Malencik and Anderson (9).

Surprisingly, MLR, VLM, and ENB induced a decrease in the fluorescence intensity of dansyl-CaM (Fig.1c-e). The quenching of dansyl fluorescence by the three peptides was associated with Ca^{++}-dependent binding to calmodulin, as the addition of 2 mM EGTA reduced the fluorescence to background values. It is possible that, upon binding to CaM, the three peptides (VLM, MLR, ENB) quench dansyl fluorescence by inducing a conformational change which results in the exposure of the dansyl group to a more polar microenvironment. This could especially be the case with VLM binding, in particular, because it induced not only a decrease in fluorescence intensity but also a red shift in λmax, both of which are indicative of an increased polarity of the microenvironment and consequent to quenching of fluorescent reporter groups (11). Although direct quenching of dansyl fluorescence by the three peptides cannot be entirely excluded at this time, quenching of fluorescent reporter groups on a number

of proteins was observed after the addition of specific substrates and has been interpreted as a result of conformational changes that were induced in the protein molecule upon binding (12-15). Kd's of 12.99 μM, 4.29 μM, and 41.26 μM were estimated for MLR, VLM, and ENB binding, respectively, by the method of Rose and O'Connell (12). The different spectral changes exhibited by the binding of MLR, ENB, and VLM to dansyl-CaM, as compared to that of CSA and GRS (Fig. 1a-e), may reflect different binding sites and/or different conformational changes induced in CaM.

Peptides that bind CaM with high affinity have been shown to inhibit the activity of CaM-dependent enzymes, particularly PDE, by competing with the enzyme for common binding domain(s) on CaM (4). Because all five peptides (CSA, GRS, VLM, ENB, MLR) were shown to bind dansyl-CaM with moderate affinity, we investigated the ability of the these peptides to inhibit PDE. As shown in Fig. 2, all five peptides inhibited PDE. Furthermore, the inhibition was CaM dependent, and the baseline activity of activator-deficient PDE was not affected by any of the peptides (data not shown). Concentrations of the peptides that inhibited PDE by 50% (IC_{50}; Fig.2) were indicative of strong to moderate inhibition(1). The IC_{50} for GRS compared well with that reported by Cox et al., (7). GRS and MLR had comparable IC_{50}'s, while those of ENB, CSA, and VLM were approximately 10 times higher (Fig. 2).

It is difficult to determine the minimum structural requirements that allow small peptides to bind to CaM and inhibit

its target enzymes. This is partly due to the relatively poor correlation between CaM binding affinity and inhibition of CaM-target enzymes. For example, it was shown that the ability of some peptides to inhibit CaM-dependent PDE activity correlates well with their respective CaM binding affinities, while other peptides which bind strongly to CaM, do not inhibit, or weakly inhibit PDE (1). In this study, the only peptide whose binding affinity correlated with its ability to inhibit PDE was GRS. Although all five cyclic peptides studied are hydrophobic, hydrophobicity and binding affinity could not be correlated. In contrast, the more amphipathic, or sided cyclic peptides, MLR and GRS, inhibited PDE more strongly than the others. Although possession of some degree of hydrophobicity may be necessary for a small peptide to interact with CaM, binding affinity and the ability to inhibit CaM-dependent enzymes may depend upon subtle molecular interactions between the peptide, CaM, and enzyme.

Fig. 1(a-e). Fluorescence emission spectra of 1 μM dansylated-CaM alone (∅) and in the prescence of increasing μM concentrations of (a) CSA, (b) GRS, (c) MLR, (d) VLM, and (e) ENB. Some samples contained dansyl-CaM (Sigma), cyclic peptide and 2 mM EGTA. All solutions contained 50 μM of MOPS [2-(N-morpholin-o)ethanesulfonic acid], pH 7.4, 200 mM KCl, 1 mM $CaCl_2$. Fluorescence was monitored on a Perkin Elmer model 650 fluorometer with slit widths set at 10. Temperature was maintained at 20°C. Emission fluorescence of dansylated-CaM was
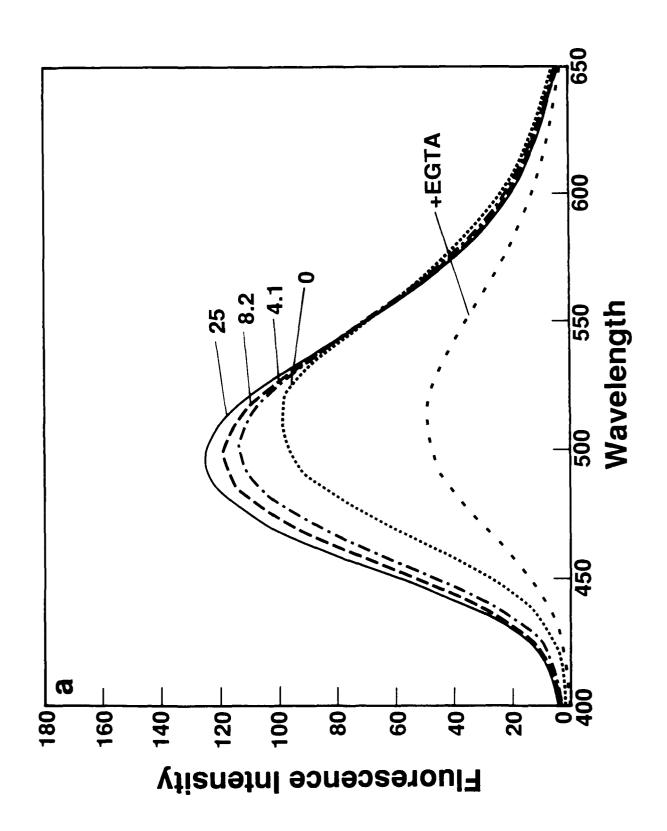
scanned from 400 to 650 nm with excitation at 345 nm. Control solutions of MOPS and cyclic peptide showed no fluorescence at the above experimental conditions.
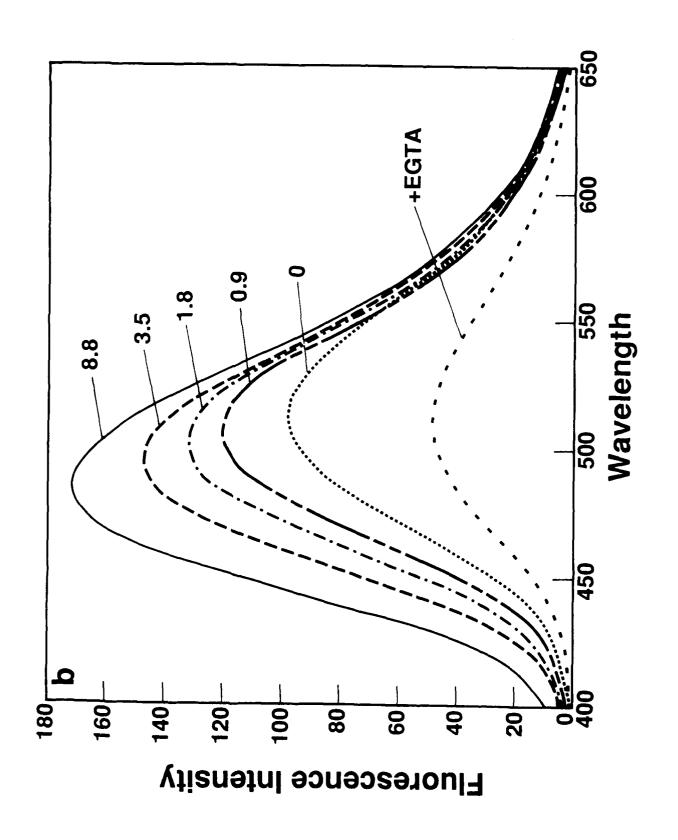
Fig. 2. Inhibition of CaM-dependent PDE activity of peptides. Percent inhibition of CaM-dependent PDE activity (absorbancy 660 nm) was plotted as a function of CSA (-□-), GRS (-O-), VLM (-●-), ENB (-■-), and MLR (-△-) concentrations. A modified assay, performed as described by Colombani et al. (6), was used to determine the activation of PDE by CaM and its inhibition by cyclic peptides. Activator deficient bovine brain 3', 5'-cyclic-nucleotide phosphodiesterase (PDE) and cAMP in 0.5 ml Tris buffer (containing 0.01 mM $CaCl_2$, pH 7.5) were incubated alone, with CaM (0.5 U), with CaM and increasing concentrations (5-5000 nM) of cyclic peptides, or with cyclic peptides alone for 20 min at 30°C. After the reaction was stopped by placing the tubes in boiling water for 2 min and then cooling them to room temperature, 5'-nucleotidase (0.05 ml, 1U), was added and the tubes were incubated at 37°C for 20 min. The reaction was stopped by adding 0.1 ml of trichloroacetic acid (10% w/v), followed by 0.1 ml of 10% molybdic acid. A blue color was developed by the addition of 0.1 ml of Fiske-Subbarow reagent (10 mg/ml) and incubated for 20 min at room temperature. Absorbance at 660 nm was measured spectrophotometrically. Half-maximal inhibition (IC_{50}) of PDE occurred approximately at 0.11 µM (MLR); 0.45 µM (GRS); and > 5 µM for ENB, CSA, and VLM .
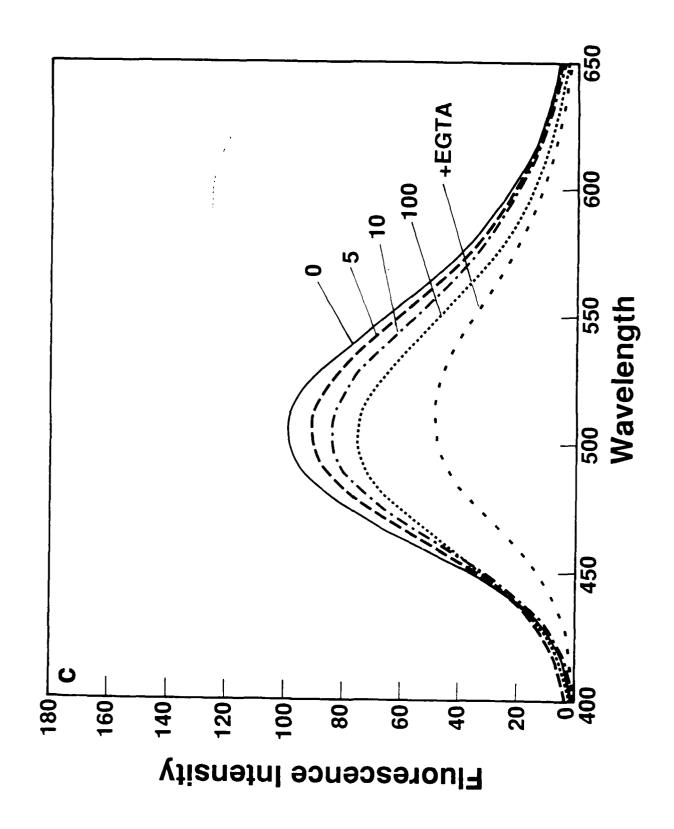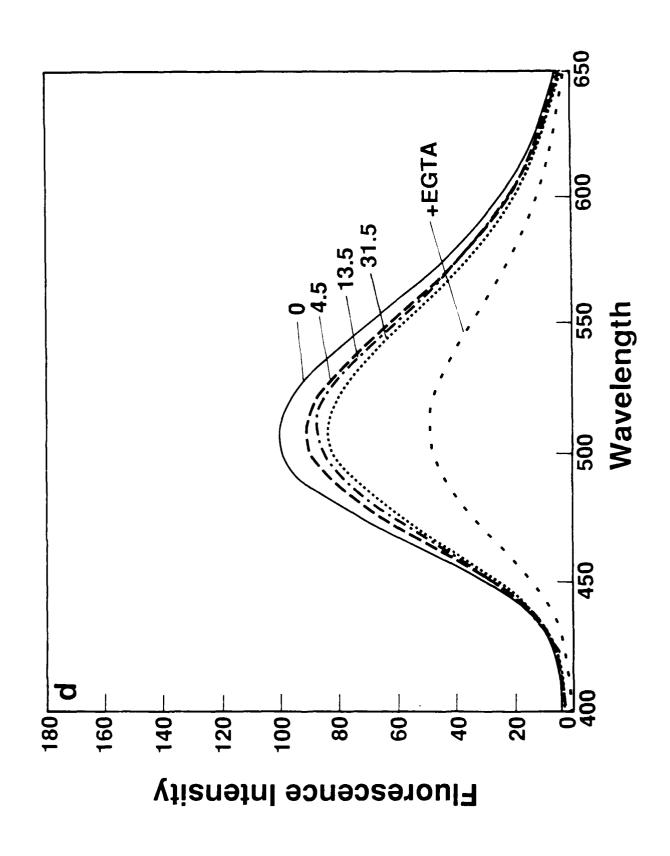
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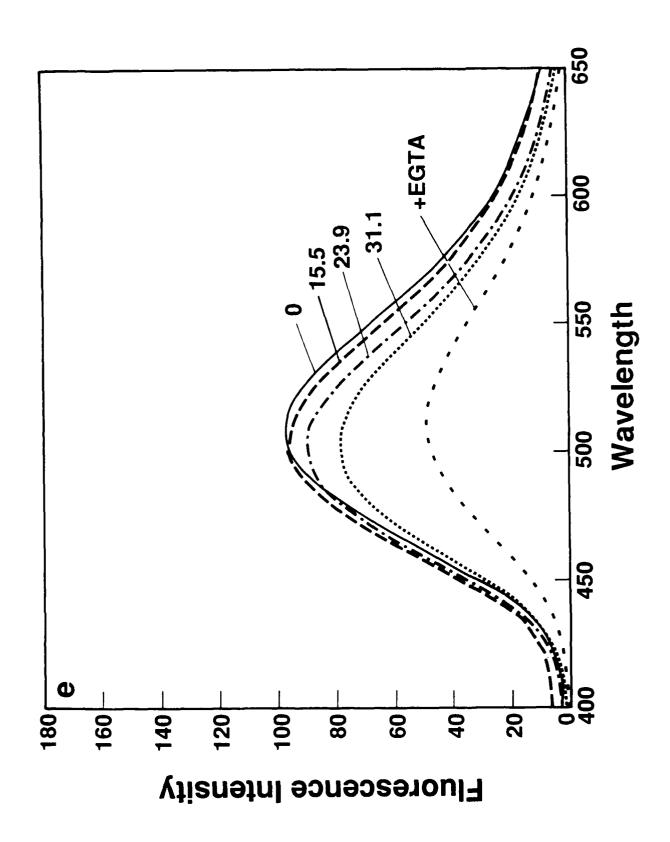
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b

Fluorescence Intensity

Wavelength

8.8

3.5

1.8

0.9

0

+EGTA

C

Fluorescence Intensity

Wavelength

0
5
10
100
+EGTA

d

Fluorescence Intensity
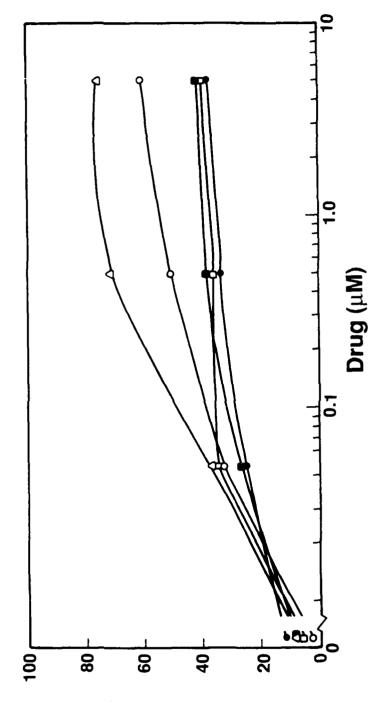
Wavelength

0
4.5
13.5
31.5
+EGTA

e

Fluorescence Intensity

Wavelength

0
15.5
23.9
31.1
+EGTA

Percent Inhibition of PDE Activity

Drug (μM)